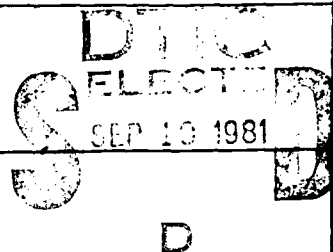LEVEL II   /3 S

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Although acquired immunity to leishmaniasis is generally considered to be cell-mediated, humoral factors may be partially responsible. The present study showed that antisera from C57Bl/6J mice superinfected with Leishmania donovani contained cytophilic antibody and opsonins for both the amastigote and promastigote stages of the parasite. When mouse hyperimmune serum was tested for cytophilic and opsonizing antibodies in an in vitro macrophage culture system, 1.5-4.0 times more parasites were bound to macrophages at

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4°C, and subsequently phagocytized at 37°C, than their respective controls.
The percentage of cells bearing or containing parasites, respectively, also
was significantly greater than their respective controls. These differences
were observed at 3, 6 and 9 days when sera from mice killed 10 or 11 days
after superinfection were used. However, when sera from mice killed 24 days
after superinfection were tested with amastigotes, at 9 days the number of
parasites and the percentage of cells parasitized decreased to or significantly
below control values. Thioglycollate-stimulated macrophages, treated with
hyperimmune serum bound more amastigotes at 4°C than those treated with
control serum; activated macrophaged demonstrated increased non-specific
binding of amastigotes. Treatment of macrophages with trypsin reduced both
cytophilic antibody-specific and non-specific binding of amastigotes. The
specific in vitro effects of anti-leishmanial antibody from superinfected
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leishmaniasis in the mouse.

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Report Number 1

Functional Role for Humoral Antibodies in Leishmaniasis in Laboratory Animals

Annual Summary Report

Robert/Herman
January 1980

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Summary:

Although acquired immunity to leishmaniasis is generally considered to be cell-mediated, humoral factors may be partially responsible. The present study showed that antisera from C57Bl/6J mice superinfected with Leishmania donovani contained cytophilic antibody and opsonins for both the amastigote and promastigote stages of the parasite. When mouse hyperimmune serum was tested for cytophilic and opsonizing antibodies in an in vitro macrophage culture system, 1.5-4.0 times more parasites were bound to macrophages at $4^{\circ}C$, and subsequently phagocytized at $37^{\circ}C$, than their respective controls. The percentage of cells bearing or containing parasites, respectively, also was significantly greater than their respective controls. These differences were observed at 3, 6 and 9 days when sera from mice killed 10 or 11 days after superinfection were used. However, when sera from mice killed 24 days after superinfection were tested with amastigotes, at 9 days the number of parasites and the percentage of cells parasitized decreased to, or significantly below, control values. Thioglycollate-stimulated macrophages, treated with hyperimmune serum bound more amastigotes at $4^{\circ}C$ than those treated with control serum; activated macrophages demonstrated increased non-specific binding of amastigotes. Treatment of macrophages with trypsin reduced both cytophilic antibody-specific and non-specific binding of amastigotes. The specific in vitro effects of anti-leishmanial antibody from superinfected mice might indicate a possible role for humoral antibody in immunity to leishmaniasis in the mouse.

Foreward:

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

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There is little doubt that in leishmaniasis of man and experimental animals, specific cell-mediated reactions greatly influence the course, degree, and probably the final outcome of the infections (see review by Zuckerman (1), Preston and Dumonde (2) and Mauel and Behin (3)). A direct role for thymus-dependent cells in acquired immunity of mice to Leishmania donovani has been suggested (4). The effect of selective immunosuppression of thymus-dependent immune responses on L. enriettii infection in guinea pigs (5) and on L. tropica infection in mice (6) indicate a protective function for thymus-dependent cells. In addition, congenital athymic mice supported higher spleen and liver populations of L. donovani than their thymus-intact littermates (7). The physiological and immunological influences upon host macrophages may also cause changes in the growth and reproduction of parasites within them (8, 9, 10, 11, 12).

In the past, however, relatively little attention has been devoted to the possible role of specific anti-leishmanial antibody as an influence in the resolution of infection, although its presence has been demonstrated in cutaneous (13, 14), American mucocutaneous (15) and visceral (16, 17) leishmaniasis. Recently, suggestions have been made that such antibody functions in acquired immunity to leishmaniasis in man (11) and mice (2, 6) infected with L. tropica.

The results of the present study suggest that, under certain conditions, specific mouse anti-leishmanial hyperimmune sera, through the action of cytophilic and opsonizing antibody, influence the binding, subsequent phagocytosis, and parasitization of macrophages by L. donovani in an in vitro system. Additionally, the effects of activation of macrophages by thioglycollate medium upon the binding of specific cytophilic antibody and the effects of treatment of macrophages by trypsin upon the antibody-mediated and non-specific binding of amastigotes was investigated.

MATERIALS AND METHODS

Hyperimmune sera. To stimulate the production of anti-leishmanial hyperimmune sera, mice (female, C57Bl/6J, purchased from Jackson Laboratories, Bar Harbor, Maine, 6-8 weeks old, 50/group) were injected intravenously (i.v.) with $0.5-1.0 \times 10^7$ amastigotes of L. donovani, 3S strain (18), in infected hamster spleen tissue (19). The same number of mice injected i.v. with an amount of normal hamster spleen tissue equal to that inoculated with parasites, served as controls. In some experiments parasites were washed several times in phosphate buffered saline (PBS), pH 7.0, counted with a Petroff-Hausser bacterial counting chamber and the number adjusted to the desired concentration with PBS. Sixty to 65 days later the mice were superinfected i.v. with the same number of amastigotes or normal hamster spleen tissue, respectively, and 10 or 11 days later 25 mice from each group were killed, blood taken by cardiac puncture and pooled by group. Spleens and livers were weighed and impression slides of these organs prepared for assessment of parasite burdens (19). The bloods were stored overnight at $4^{\circ}C$, centrifuged for the collection of sera which were stored at $-70^{\circ}C$ in 0.5 ml aliquots. At 24 days after superinfection, the remaining 25 mice of each group were killed and the bloods processed, as before, for sera.

Macrophage culture. In initial experiments 160 x 15 mm plastic petri dishes (Falcon Plastics, Oxnoid, Cal.), each containing 3 glass coverslips (18 mm diameter), were used; in later experiments eight-chambered Lab-Tek culture slides (Lab-Tek Products, Div. Miles Laboratories, Inc., Westmont, Ill.) were used. Both methods served equally well except that the use of Lab-Tek slides required fewer total macrophages and was more conservative of the amount of serum needed for testing.

For initiation of macrophage cultures, cells from the unstimulated peritoneal cavities of normal female C57B1/6J mice were harvested by Pasteur pipette following the inoculation of 3 ml of Tissue Culture Medium 199 (Grand Island Biologic Co., Grand Island, N.Y.) (M199) containing penicillin (100 units/ml) and streptomycin (100 µgm/ml), and ammonium heparin (Sherwood Medical Industries, St. Louis, Mo.) (10 units/ml). The cell suspensions from several mice were pooled, centrifuged at 400 x g for 10 minutes, and the pellet resuspended in M199 containing antibiotics and 20% heat-inactivated fetal bovine serum (FBS) (Microbiological Associates, Walkersville, Md.), but no heparin (complete medium), to 4 times the original volume. Wells of several Lab-Tek slides were each inoculated with 0.5 ml of the cell suspension. All slides were then incubated overnight at 37°C in 5% CO_2 in air after which one slide was rinsed in PBS, air dried, fixed in absolute methanol and stained with Giemsa. The mean number of cells/well, determined by counting cells in a representative number of fields of at least 4 wells, was calculated in a manner similar to that described previously (20), and was found to range from 5.5×10^4 to 8.3×10^4. When petri dish cultures were used, the pooled peritoneal cell suspension from mice was brought to twice its original volume with complete medium and 4 ml inoculated/dish. For treatment of macrophages with sera, individual coverslips were removed from their original dishes and placed into new ones before sera were applied. In all experiments in which coverslips were used, they were removed from dishes, air dried, fixed in methanol, stained with Giemsa and mounted on slides. The phagocytic capacity of macrophages in culture, either on coverglasses in petri dishes or in wells of Lab-Tek slides, was proven by the addition of 1.1 µm latex polystyrene beads (Coulter Electronic, Inc., Hialeah, Fla.); greater than 95% of cells contained beads after incubation for one-half hour at 37°C.

Preparation of amastigotes of L. donovani. A suspension of amastigotes was prepared by triturating a heavily infected hamster spleen in 10 ml of M199 (containing penicillin and streptomycin) in a Ten Broeck all-glass tissue grinder. The ground spleen was then centrifuged at $4^{\circ}C$ at 75 x g for 5 minutes and the supernatant, containing a large number of parasites, was recovered and the parasites washed twice at 2,700 x g in the same medium. The final pellet of parasites was resuspended in 10 ml of medium and the parasites counted in a Petroff-Hausser counting chamber. The number of amastigotes was then adjusted to a multiplicity of 0.5-10 (in a volume of 0.5 ml) of the number of macrophages/well.

Test for cytophilic activity of hyperimmune sera. All sera were used inactivated ($56^{\circ}C$ for 30 minutes) and diluted 1:3 or 1:4 with M199. For testing, wells of several Lab-Tek slides, containing macrophage cultures implanted one day earlier, were rinsed twice with warm ($37^{\circ}C$) M199 (containing antibiotics) and then 0.1-0.2 ml of hyperimmune serum from mice killed 10 or 11 days or 24 days following superinfection was added to each of 4 wells of several slides; the same volume of control serum was applied to the remaining 4 wells of each slide. All slides were then incubated at $37^{\circ}C$ in 5% CO_2 in air for one-half hour after which all wells were rinsed twice with warm M199. Amastigotes (0.5 ml), prepared as described above, were inoculated into each well and all slides were then incubated at $4^{\circ}C$ for 1 hour to allow parasites to bind to macrophage surfaces. All wells were then rinsed 3 times with M199; one slide was fixed and stained with Giemsa. To all the wells of the remainder of the slides were added 0.5 ml complete medium (i.e., containing 20% FBS) and all slides were incubated at $37^{\circ}C$ in 5% CO_2 in air. One hour later, one slide was removed, air dried, fixed and stained with Giemsa to assess the extent of phagocytosis of

the parasites. Thereafter, slides were fixed and stained at 3, 6 and 9 days with the medium being changed at these times on remaining slides. In some experiments promastigotes, rather than amastigotes, were used, but only with hyperimmune serum from mice killed 10 days after superinfection.

Test for opsonizing activity of hyperimmune sera. To assess the opsonizing capacity of hyperimmune sera, numbers of amastigotes, in volumes calculated to be sufficient for several slides (0.5 ml/well), prepared in accordance with the mean number of macrophages/well, as described above, were centrifuged at 2,700 x g for 10 minutes. Separate pellets of parasites were resuspended in 1 ml of hyperimmune and control sera, respectively, for one-half hour at $37^{\circ}C$ in 5% CO_2 in air, the parasites then being washed 3 times in M199 (containing anti-biotics). Both hyperimmune serum- and control serum-treated parasites were then resuspended in complete medium to their original volumes and 0.5 ml of hyperimmune serum-treated amastigotes were inoculated into 4 wells of each of several Lab-Tek slides and 0.5 ml of control serum-treated amastigotes into the remaining 4 wells of each slide. These were incubated at $4^{\circ}C$ and the procedure, as described above, was followed. As in the tests for cytophilic antibody, in some experiments promastigotes, rather than amastigotes, were used but again, only with hyperimmune serum from mice killed 10 days after superinfection.

Activation of macrophages. Thioglycollate-stimulated macrophages were obtained from the pooled peritoneal exudates of each of 3 C57Bl/6J mice injected with 2 ml of thioglycollate medium 3 days before the cells were harvested; control macrophages were obtained from mice which had received no injections. Cell suspensions from thioglycollate-injected and control mice were adjusted to 1 x 10^6 cells/ml with complete medium, explanted to Lab-Tek slides and incubated

at $37^{\circ}C$ in 5% CO_2 in air for 1 hour. The slides were then rinsed in warm M199 and the cytophilic capacity of hyperimmune serum from mice killed 10 days following superinfection, and control serum, were tested.

Trypsinization of macrophage cultures. To test whether binding sites for cytophilic antibody on macrophages are trypsin-sensitive, peritoneal macrophages (from female BALB/c mice, Charles River Breeding Laboratories, Inc., Wilmington Mass.) were harvested as usual, but resuspended in M199 without FBS and used the same day as explanted. These experiments were carried out in petri dishes, each containing 3 coverslips on which macrophages were maintained. Cultures to be tested were treated with 4 ml of trypsin (500 µgm/ml) in Hanks' BSS for 45 minutes; control cultures were treated with Hanks' BSS only. All coverslips were then rinsed well in a beaker of Hanks' BSS before the sera were applied. In these experiments, hyperimmune sera obtained from BALB/c mice 10 days after superinfection were used. Amastigotes, in a multiplicity of 10 with reference to numbers of macrophages, were then applied to all cultures for 1 hour at $4^{\circ}C$ and the degree of binding of the parasites to the macrophages was assessed from Giemsa-stained slides.

In other experiments, the effect of trypsin on the non-specific binding of amastigotes to macrophages was tested. These experiments were executed in the same way, except that sera were not used. Since trypsin may become inactivated by prolonged exposure to cells, cells were treated sequentially with trypsin. Cultures were treated with 1, 2, or 3 exposures to trypsin (500 µgm/ ml, 4 ml/dish) for 45 minutes at $37^{\circ}C$ in 5% CO_2 in air. After each exposure the coverslips were rinsed in Hanks' BSS and new trypsin applied, where required. One culture, not exposed to trypsin served as a control and another, exposed to trypsin for one 45 minute period, was used to determine whether binding sites

were regenerated after reincubation of the cells for 4 hours at 37°C in complete medium. At the appropriate times, amastigotes were added, in a multiplicity of 10, and the cultures incubated at 4°C for one-half hour for binding of amastigotes to macrophage surfaces.

Promastigotes. Promastigotes were grown in tubes of Tobie's diphasic medium (21) modified in that Minimal Essential Medium (MEM) (Grand Island Biological Co.) containing penicillin and streptomycin (100 units/ml and 100 ugm/ml, respectively) was used as the liquid overlay.

In all experiments of this study, the assessment of the numbers of parasites bound to, or phagocytized by, macrophages was evaluated following the observation of at least 200 randomly encountered macrophages on coverslips and wells of Lab-Tek slides. This was done independently by two observers using coded slides. Statistical analysis of the data was by 2-way analysis of variance.

Fluorescent Antibody Technique. Macrophage cultures were incubated with hyperimmune anti-leishmanial or control serum in varying dilutions (in PBS or M199) at 37°C for one-half hour. Each 0.5 ml of serum had been adsorbed twice, for 10 minutes each time, with 50 mg of normal hamster spleen tissue in 0.1 ml PBS. The cultures were then rinsed 3 times with PBS and 2-3 drops of fluorescein-conjugated rabbit anti-mouse immunoglobulin (IgG) (diluted 1:5 with PBS) (Cappel Laboratories, Inc., Downington, PA.) was added to each well and incubated for 30 min. at room temperature. The cultures were then rinsed 3 times (10 min. each) with PBS, overlaid with phosphate buffered glycerol (pH 7.2) and observed under a coverslip with a Leitz Ortholux II microscope equipped with a Ploem illuminator and "I" filter cube. Hyperimmune sera from mice killed 10 or 11 days and 24 days after superinfection were tested.

RESULTS

Effects of hyperimmune sera on binding and subsequent phagocytosis of

L. donovani by macrophages. Mouse macrophages, following incubation with

anti-leishmanial hyperimmune sera, obtained from mice 10 or 11 days following

superinfection with amastigotes of L. donovani, bound a statistically significantly

greater number of parasites, as either amastigotes (Fig. 1A) or promastigotes

(Fig. 1B), to their surfaces at $4^{o}C$ when compared to cultures treated with sera

from mice injected with normal hamster spleen tissue. When these cultures were

rinsed, complete medium (i.e., containing FBS) added, and the macrophages allowed

to phagocytize parasites bound to their surfaces for 1 hour at $37^{o}C$, hyperimmune

serum-treated macrophages contained a significantly greater number of amastigotes

than did controls. These differences were also observed at 3, 6 and 9 days

(Fig. 2). Similarily, the mean percentage of infected cells (Fig. 3) as well

as the mean number of amastigotes/infected cell (Fig. 4) were also significantly

greater in cultures which had been treated with anti-leishmanial hyperimmune

serum. These same effects were observed when parasites, as either amastigotes

or promastigotes, were opsonized prior to being added to macrophage cultures.

When sera from mice killed 24 days after superinfection were tested, results

through the 6th day of culture were similar to those observed when 10 or 11

day sera were used. However, 9 day macrophage cultures which had been treated

with anti-leishmanial hyperimmune serum from mice killed 24 days following

superinfection showed significantly fewer amastigotes/200 cells, % of cells

infected and amastigotes/infected cell when compared to controls (inserts of

Fig. 2, 3, and 4, respectively) in 3 of 4 experiments. These reversals seen

at this time were also noted when amastigotes were opsonized; promastigotes were

not tested. Mean spleen parasite burdens of superinfected mice decreased
between 10 or 11 days and 24 days from 6.6×10^6 to 1.7×10^6 and, for the
liver, from 6.0×10^7 to 3.1×10^7. These experiments were carried out 4 times:
twice with 10-day sera and twice with 11-day sera; 4 times with 24-day sera.
Different batches of sera were obtained from mice at the different times,
once from mice killed 10 days and once from those killed 11 days after superin-
fection and twice from mice killed 24 days after superinfection, all gave
reproducible results. In all experiments, differences between comparable groups
(see Results) were statistically significant (i.e., between $p < .05$ and $< .0025$)
at most times.

Thioglycollate-stimulated macrophages. Thioglycollate-stimulated peritoneal
macrophages, following treatment with hyperimmune anti-leishmanial serum, did
not bind, at $4^\circ C$, a greater number of amastigotes than did similarly treated
peritoneal macrophages from non-stimulated mice, when compared to their respective
controls (Fig. 5). Thus, macrophages from non-stimulated mice which had been
treated with hyperimmune sera bound 2.1 times more amastigotes/200 macrophages
than did those treated with serum from hamster spleen-injected mice ($p < .0025$)
while those from stimulated mice bound 1.7 times more than their controls
($p < .0025$). Similarly, there were 2.0 times more cells infected in cultures
from non-stimulated, and 1.6 times more in cultures from stimulated mice, both
treated with hyperimmune serum, when compared to their respective controls
(in both cases, $p < .0025$). Although more amastigotes bind to macrophages
from thioglycollate-stimulated mice after treatment with either hyperimmune or
control sera when compared to similarly treated macrophages from non-stimulated
mice, the differences within each group were about the same. This relationship
is observed also when the % of cells infected are compared (Fig. 5).

Effects of treatment of macrophages with trypsin on binding of amastigotes
of L. donovani. Treatment of macrophage cultures with trypsin before the
addition of anti-leishmanial hyperimmune serum significantly reduced the number
of parasites bound at $4^{\circ}C$, when compared to cultures not treated with the
enzyme (p < .005). Trypsin treatment also significantly reduced the number of
parasites bound after treatment with contol serum (p < .0125) (Fig. 6). Similarly,
trypsin treatment also significantly reduced the % of cells infected in hyper-
immune and control serum-treated cultures when compared to non-trypsin-treated
cultures, respectively (p < .0025 and p < .0125).

When cultures of macrophages were treated sequentially with trypsin, the
capacity of amastigotes to bind to macrophage surfaces decreased with each
trypsin treatment. Non-treated macrophages bound a mean of 53 amastigotes/200
macrophages while after 1, 2, and 3 trypsin treatments these values were 33,
21, and 10, respectively. Amastigotes bound to 20.3% of the non-treated
macrophages while to those macrophage cultures treated with trypsin once,
twice and 3 times, the % of cells bearing amastigotes were 13.8, 8.0 and 4.6,
respectively (Fig. 7). When macrophage cultures were allowed to recover for
four hours following a single trypsin treatment and then tested for amastigote-
binding at $4^{\circ}C$, the number of amastigotes bound to macrophages and the % of cells
to which amastigotes were bound were almost the same as those of controls (Fig. 7).

Detection of cytophilic antibody with fluorescein-conjugated IgG. Hyper-
immune serum from mice killed 10 or 11 days and 24 days following superinfection
showed cytophilic antibody on macrophage surfaces (Fig. 8); no such antibody
could be detected in the respective control sera.

DISCUSSION

The results of these experiments clearly indicate that sera from mice superinfected with L. donovani demonstrate specific cytophilic and opsonizing capacities when tested in vitro in macrophage culture. In parasitic and other infections in which humoral antibodies are known to serve a protective function, any increase in the phagocytosis of the etiologic agent through the action of humoral antibody is understandably beneficial to the host. Among some protozoan parasitic infections, increases in phagocytosis by immune serum, as in rodent malaria (22, 23), have been shown. In Trypanosoma cruzi infection in mice, enhanced protection by phagocytosis (24) is probably implemented through specific antibodies (25, 26). A similar mechanism probably functions in immunity in some other experimental trypanosome infections also (27, 28).

In leishmaniasis, however, that serum from mice, acting in either a cytophilic or opsonic capacity, should enhance intracellular parasitization in vitro when spleen and liver parasite burdens are decreasing (between 10 or 11 days and 24 days after superinfection) is paradoxical, since this parasite occurs, in vivo, only within phagocytic cells of the reticuloendothelial system. It may be envisaged, however, that such an enhancement of intracellular parasitization in vitro is a prelude to the parasites' destruction, either through an antibody-dependent cell-mediated cytoxicity (29, 30), a modification of which might help to explain the cytophilic antibody-mediated destruction of promastigotes of L. tropica in vitro (11), or through a mechanism in which the activation of macrophages is invoked through contact with sensitized lymphocytes (Bradley, personal communication to Mauel and Behin (3)). It is interesting that Bradley's "...experimental design did not exclude antibody from being an essential component of the reaction." (3). Indeed, the thought that cell-bound antibody

may actually influence the cellular response in leishmaniasis has been expressed (31), such antibody functioning, perhaps, to aid the presentation of antigen to T lymphocytes (32).

Because opsonizing and cytophilic activity have been demonstrated in sera from mice 10 or 11 days after superinfection with L. donovani, these separate functions may be attributed to different components of the serum (33, 34) which may act in concert to help change the course of infection in vivo, possibly in a manner not unlike the synergistic relationship of opsonizing and cytophilic antibody observed in rat anti-malarial serum (35). It is possible that, with increasing time after superinfection, the functional capacity of either or both of these antibodies may vary, resulting in the changes observed at 9 days of culture (see inserts of Fig. 2, 3, and 4). Actually, changes may occur before 9 days, so that the viability of the parasites actually decreases prior to their reduction in number (10).

Although thioglycollate-stimulated macrophages have a greater number of Fc receptors on their surfaces than do macrophages from non-stimulated mice (36), the relationship between numbers of amastigotes bound to hyperimmune serum-treated thioglycollate-stimulated and normal macrophages, and the percentages of cells bearing amastigotes on their surfaces, were similar when compared to their respective controls (Fig. 5). In comparison to cultures from non-stimulated mice, the increased binding of amastigotes and percentage of cells bearing them in cultures of macrophages from thioglycollate-stimulated mice could be due to entrapment of parasites by the very obvious extensive cytoplasmic processes characteristic of thioglycollate-stimulation. It could also be due to functional changes of C3 receptors (37) or to an increase in non-immunologic, non-specific receptors (38).

Mouse IgG_{2a} binds strongly to the macrophage surface (36) by trypsin-sensitive Fc receptors (36, 39). Thus, the inhibition of cytophilic antibody-mediated binding of amastigotes to macrophages and the decrease in the percentage of macrophage-bearing parasites (Fig. 6) is most likely due to the ablation of Fc receptors by trypsin. Other trypsin-sensitive receptors on plasma membranes of leucocytes are those for C3 (37) which, in unstimulated mouse macrophages, are not associated with the cells' phagocytic mechanisms, but function only to bind C3-coated particles to the cell's surface (37). The possibility exists that non-cytophilic antibody-mediated binding of amastigotes to macrophages is by macrophage Fc receptors for immunoglobulin on the amastigote surface and/or by binding of complement to such antigen-antibody complexes. The reduced binding of amastigotes to trypsin-treated cells, and the reduced percentage of cells binding them after application of hyperimmune sera (Fig. 6), or in its absence (Fig. 7), could be a result of the trypsin-sensitivity of Fc binding sites as well as the C3 receptors. When promastigotes, which are grown in culture, are used to test the cytophilic and opsonic capacities of hyperimmune sera, these possibilities are obviously precluded for this stage of the parasite. However, the binding of amastigotes and promastigotes to macrophage membranes could possibly be due, in part, to the existence of other macrophage recognition sites (38).

The increased number of parasites/200 cells and the increased percentage of infected cells observed in almost all experiments after one hour's incubation at $37^{\circ}C$, compared to numbers bound to macrophages at $4^{\circ}C$, may be due to the capacity of amastigotes to adhere to glass. These parasites are not removed by washing of the culture wells, "free" parasites (i.e., parasites not associated with a cell) often being observed after incubation at $4^{\circ}C$ but are infrequently

seen after incubation at 37°C. Thus, amastigotes adhering to the glass slide at 4°C may dislodge at 37°C and be phagocytized. Like Chang (40), little, if any, reproduction of amastigotes was observed over a 9 day period; uneven distribution of parasites within macrophages (41) was seen regularly.

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LEGEND FOR FIGURES

Figure 1. Specific binding of (A) amastigotes and (B) promastigotes of
Leishmania donovani, in vitro at $4^{\circ}C$, to mouse macrophages previously
treated with anti-leishmanial hyperimmune antiserum from mice 10 or
11 days or 24 days following superinfection (x 1,250).

Figure 2. Binding of amastigotes of L. donovani to macrophages in vitro at
$4^{\circ}C$, their subsequent phagocytosis at $37^{\circ}C$ for 1 hr, and the parasitiza-
tion of macrophage cultures for 9 days (± S.E.). ▨ Macrophage
cultures treated with anti-leishmanial hyperimmune serum obtained from
mice 10 or 11 days following superinfection (± S.E.). ☐ Macrophage
cultures treated with serum from mice injected with normal hamster spleen
tissue (± S.E.). Insert: numbers of amastigotes/200 macorphages at 9
days in cultures previously treated with anti-leishmanial hyperimmune
serum obtained from mice 24 days following superinfection. The differences
between values for hyperimmune serum- and control serum-treated cultures
at each time are significantly different from each other ($p < .025$ at
6 and 9 days and for insert; $p < .0125$ at 3 days and $4^{\circ}C$ (1 hr.);
$p < .0025$ at $37^{\circ}C$ (1 hr.).

Figure 3. Percentages of macrophages to which parasites were bound in vitro
at $4^{\circ}C$ and which contained intracellular amastigotes thereafter (± S.E.).
All conditions are the same as indicated for Figure 2 ($p < .05$ at 6 days;
$p < .025$ at $37^{\circ}C$ (1 hr.); $p < .0025$ for insert).

Figure 4. Parasites/infected macrophage. The conditions are the same as
indicated for Figure 2 ($p < .05$ for insert and at $4^{\circ}C$ (1 hr.); $p < .0125$
at 3 and 6 days; $p < .0025$ at $37^{\circ}C$ (1 hr.)).

Figure 5.   Binding of amastigotes of L. donovani, in vitro at 4°C, to macrophages

from thioglycollate-stimulated and non-stimulated mice treated with

anti-leishmanial hyperimmune or control serum (± S.E.).   A.   Macrophages

from non-stimulated mice.   B.   Macrophages from thioglycollate-stimulated

mice. ⬚ Cultures treated with anti-leishmanial hyperimmune serum. ⬚

Cultures treated with control serum.   In each case, when cultures treated

with the different sera were compared, both the number of amastigotes/200

macrophages and the % of mcarophages binding amastigotes differed

significantly (p < .0025).

Figure 6.   Binding of amastigotes of L. donovani, in vitro at 4°C, to trypsin-

treated and non-treated macrophages (± S.E.).   A.   Non-treated macrophages.

B.   Trypsin-treated macrophages. ⬚ Cultures treated with anti-leishmanial

hyperimmune serum. ⬚ Cultures treated with control serum.   For cultures

not treated with trypsin, when hyperimmune serum- and control serum-treated

cultures were compared, both the number of amastigotes/200 macrophages

and the % of cells binding amastigotes differed significantly (p < .005

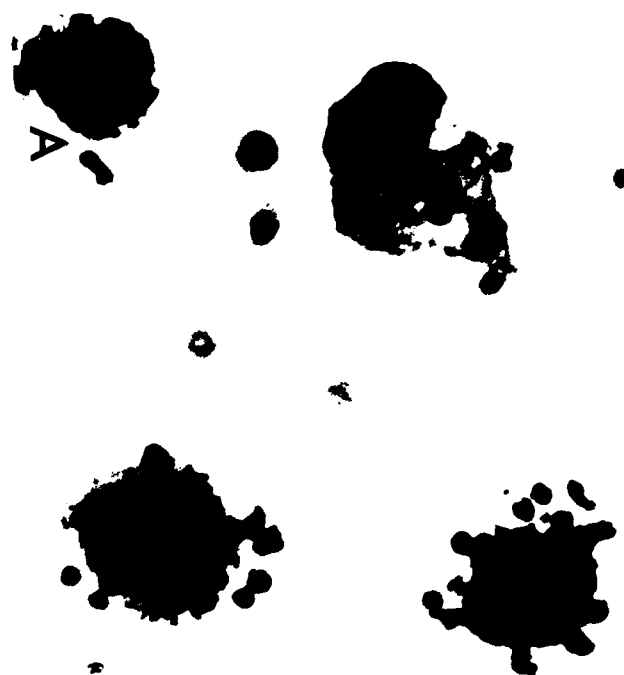and p < .05, respectively).   After treatment with trypsin, p > .05 in

both cases.

Figure 7.   Effects of sequential trypsin treatment of macrophages on the binding

of amastigotes of L. donovani (± S.E.).   0, 1x, 2x, 3x: macrophage

cultures untreated, treated once, twice or thrice with trypsin, respec-

tively.   R: macrophage cultures treated once with trypsin, washed, and

then allowed to recover for 4 hours before the application of amastigotes

for binding at 4°C in vitro.

Figure 8.   Detection of cytophilic antibody by indirect fluorescent antibody

test.   A.   Macrophages treated with anti-leishmanial hyperimmune serum

(1:8) were then exposed to fluorescein-conjugated rabbit anti-mouse IgG (x 938).

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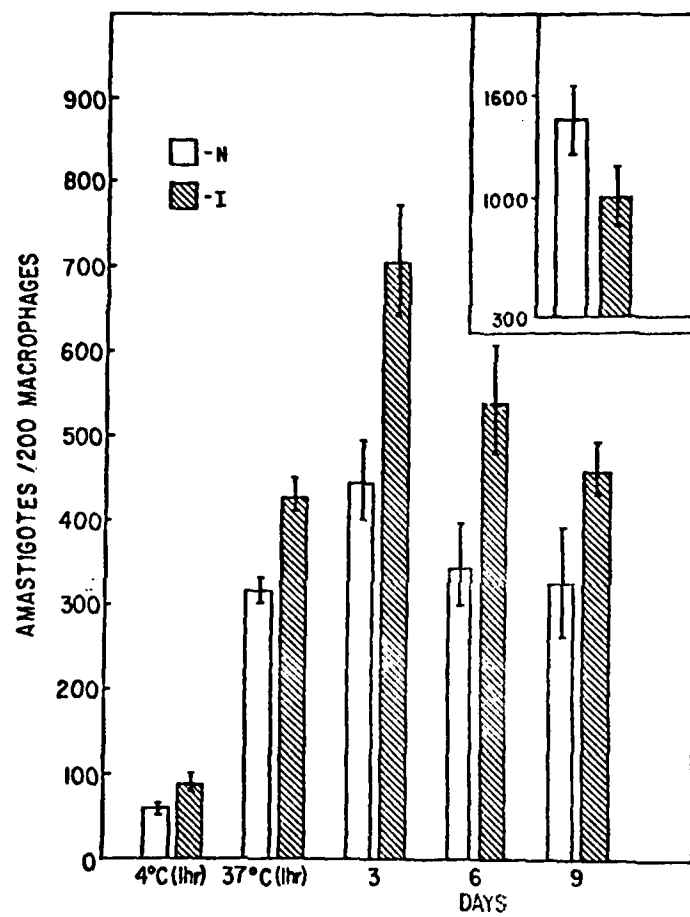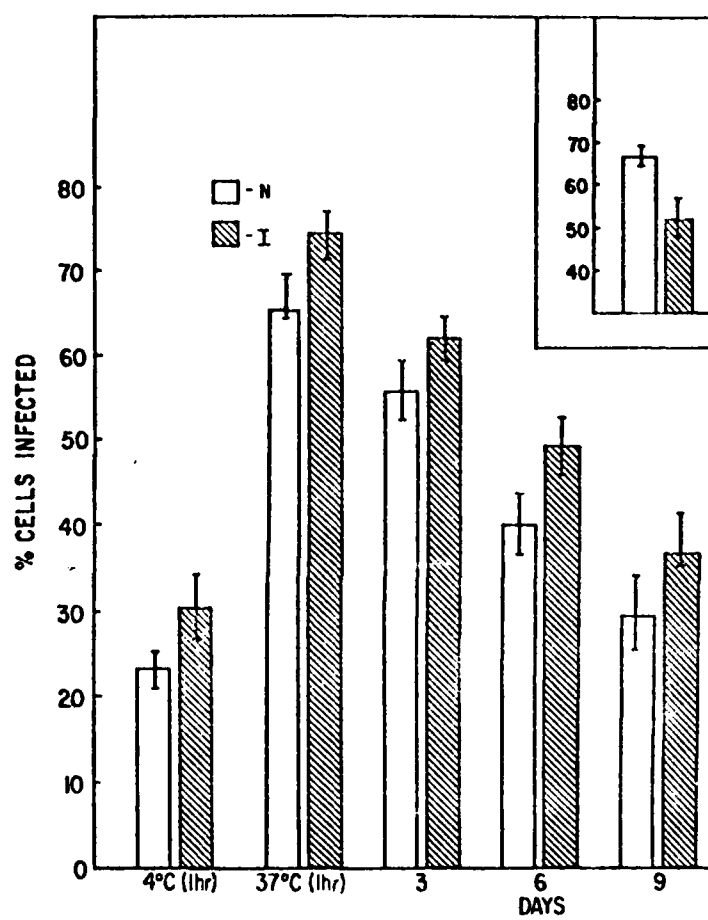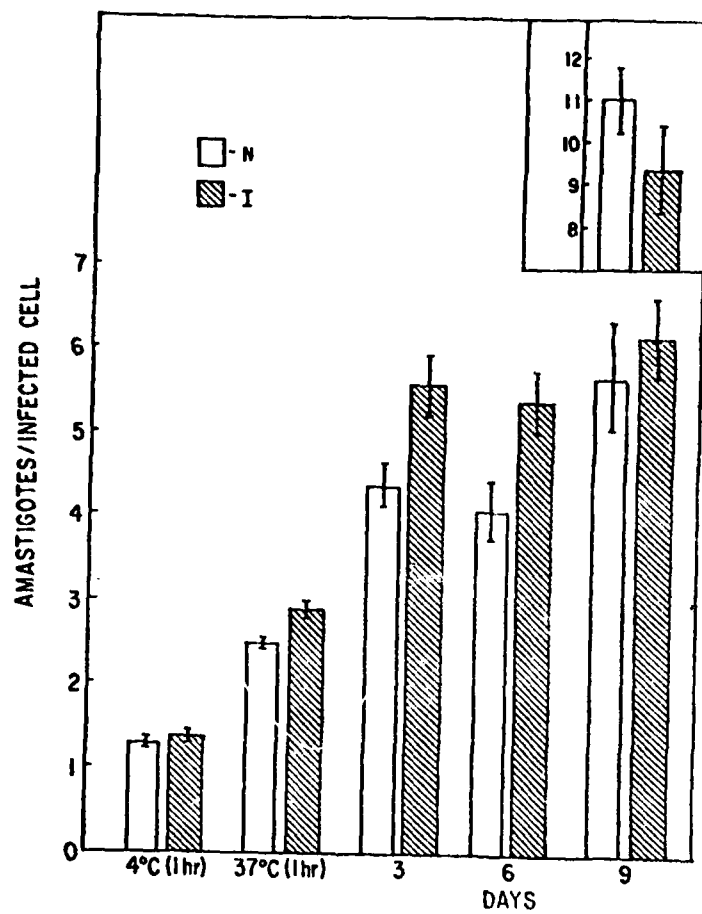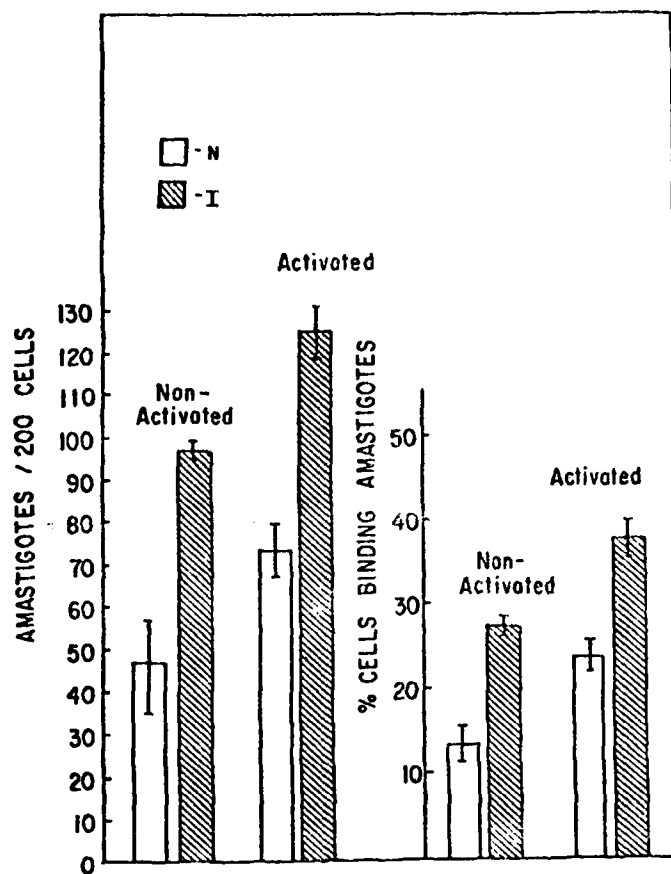   Fort Sam Houston, TX 78234

Figs. 1A and 1B

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7